Advanced hardware and software tools for fast multidimensional imaging of living cells

Jason R. Swedlow[1]

Wellcome Trust Centre for Gene Regulation and Expression, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

Live cell imaging is now used across all domains of cell and developmental biology (1). Combined with the straightforward facility for molecular labeling via fluorescent proteins and other labeling techniques, live cell imaging gives unparalleled access to the dynamics of ions and molecules in living cells and the patterns of growth and movement of cells in living tissues. Many critical insights into the biology of the cells of microorganisms, plants, and animals have been revealed through live cell imaging, and its applications only grow with every new labeling tool or imaging modality that is reported. Despite this success, live cell imaging is technically demanding and is a technique that has substantial limitations. Microscopy has recently enjoyed great success in breaking well-known optical limits in spatial resolution (2, 3), but for studies of dynamics in live cells, temporal resolution is at least as important—temporal aliasing causes significant artifacts and can hide biologically important phenotypes and mechanisms (4).

One other major challenge is the extreme photosensitivity of living cells. Merely exposing fungal and human cells to blue light arrests cell proliferation through absorption of various endogenous, weakly fluorescent cellular molecules (5). Introducing the fluorescent proteins or small-molecule fluorophores commonly used to mark cells and tissues only increases this sensitivity by at least an order of magnitude, because there is an increased number of molecules in excited states and thus increased generation of reactive species during relaxation from the excited state. Typical room light exposes cells to approximately 1 pW/cm^2. A conventional wide-field microscope using a white-light mercury Hg arc lamp and conventional fluorescence filters exposes the same sample to approximately 100 $\mu W/cm^2$. The power levels are substantially higher for confocal microscopy and for all modes of scanning and confocal microscopy (6).

Optimizing signal-to-noise ratio (SNR) while maintaining cell viability is often the key challenge in a live cell imaging experiment. In PNAS, Carlton et al. (7) make a series of major advances in all of these domains—they report a unique platform for rapid live cell imaging, a rigorous test of cell viability, and an application of well-established noise filtering tools to live cell imaging data. In one article they have made a number of critical steps, and it is likely the field will never be the same.

A New Platform for Light Microscopy

The first major achievement Carlton et al. (7) report is the design and implementation of the OMX microscope. Many new modalities of microscopy have been recently described and demonstrated. OMX provides a flexible platform for the development of new modes of imaging, and the authors recently reported the use of OMX to implement 3D structured illumination microscopy (8). In their most recent paper,

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Carlton et al. report the application of OMX to high-speed live cell imaging (7), but the platform lends itself easily to the application of 3D structure illumination microscopy, photoactivation light microscopy, and total internal reflection microscopy and potentially can be used for digital light sheet microscopy (9) as well as many others.

A number of key advances have been implemented in the OMX platform. There is a comprehensive improvement in physical stability using materials with very low thermal expansion coefficients; kinematic mounts for the objective lens, stage, and the "drawer" that holds the dichromatic mirrors; and acoustic and thermal isolation. For fluorescence microscopy, the light path has been optimized to minimize stray excitation light and thereby reduce a critical source of noise in most fluorescence microscopes. The system can record up to four independent fluorescence channels simultaneously. Finally, the illumination and sample holding and focusing mechanisms have been optimized for speed so that millisecond exposures are routine and microsecond exposures are possible. The stability and speed of OMX make it a great platform for building new modalities of microscopy and critically testing their performance.

In this report, Carlton et al. (7) have used the OMX platform to study the application of fast live cell imaging and specifically study the viability of cells during illumination in live cell imaging experiments. The authors use a strain of the budding yeast *Saccharomyces cerevisiae* that has two chromatin "marks" made by expressing lacI fused to GFP (GFP-lacI) in a yeast cell that includes two arrays of binding sites for lacI. When the arrays are bound by GFP-lacI, two diffraction-limited spots are created in the cells that can be followed in a fluorescence microscope. This technology is well established and now a standard methodology to study the dynamics and properties of chromatin in living cells (10, 11). Carlton et al. (7) use this system for a conceptually simple experiment: by recording 3D stacks over time at different levels of illumination and then examining the viability of cells after the imaging experiment, they measure the correlation between light dose and photodamage in their specimens. Carlton et al. (7) use a very common viability criterion, namely the ability of cells to complete division, but they extend this requirement and demand that cells complete a total of five cell divisions after the completion of the imaging experiment. This is quite a rigorous requirement, but their data clearly show that moderate levels of fluorescence illumination can arrest cell division failures two or even three cell cycles after imaging. Thus, their first major insight is that photodamage in a live cell imaging experiment can cause deleterious effects many hours after exposure to illumination has ceased. The authors then successfully reduce illumination to the point at which cell viability is not affected according to their rigorous standard, enabling sampling rates faster than most conventional microscopes. Unfortunately, the achieved SNR under this imaging regime is so low as to make the images extremely hard to interpret—it is very hard to see anything at all in their data—and this sets the stage for the next advance of Carlton et al. (7).

Revealing the Invisible

Computational image processing and analysis are now standard tools in

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[1]E-mail: jason@lifesci.dundee.ac.uk.

microscope imaging in cell biology. One of the first applications of computational image processing involved signal restoration via iterative deconvolution, which improved contrast by compensating for image blurring due to the limited aperture of the microscope objective lens. A range of methods were developed and used, and they helped advance the use of 3D imaging in cell biology and reveal the structures of cells and their constituents (12–14). However, the last few years have brought a fundamental change in the use and application of computational tools in biological microscopy. There are now a range of applications that go beyond simply enhancing a signal but in fact reveal patterns, structures, and dynamics that are not at all visible to the human eye. Fluorescence speckle microscopy uses sparsely labeled polymers and sophisticated particle tracking techniques to reveal polymer dynamics in living cells that cannot be directly appreciated by human vision (15). Applications of machine learning to cell biology now provide tools for automatically classifying images and identifying structures in them (16, 17). Image-based screening systems, or high content screening (HCS), generate many thousands or millions of images while sampling the effects of a library of small molecules or siRNAs. The scale of each HCS experiment makes reliable human observation and interpretation impossible and is another case in which automated object recognition and phenotype classification must be used to extract information from an imaging experiment (18). New methods like photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) create an image through the reconstruction of the computed positions of many individual fluorophores in a field (19). Following in this vein, Carlton et al. (7) apply image denoising algorithms that have found application in many other fields to the problem of live cell images with severely limited SNR recorded under conditions that are consistent with cell viability. This class of denoising algorithms scans the image with a small "patch" (e.g., a 5×5 grid) and defines the noise models within that patch. The algorithms can scan across space and time and look for regions with similar noise characteristics. The general premise is that regions with similar noise characteristics will be from similar parts of the sample. Having identified these similar regions, averaging can be used to smooth out some of the noise and detect objects that are otherwise not visible. The images recorded under the most restrictive illumination conditions—in which almost no structure can be recognized—reveal clear images of the chromatin dots in the cells that were imaged. Carlton et al. (7) go on to show that these methods can be applied not only to samples with single isolated points but also to much more complex structures, suggesting that there may be general methods for extracting structures from images with severely limited SNR.

The general point here is not that denoising algorithms should be used in every application of live cell imaging—like any other method, they have their limitations and can create artifacts, especially in images with very low SNR, and it is not clear from the analysis of Carlton et al. (7) whether their approach smoothes rapid motions near or at the limit of their temporal sampling. Rather, this is a reminder of the power that computational tools bring to SNR-limited data in general, and especially to live cell imaging experiments, and that increasingly, real insights from biological systems depend on the continued development and application of unique imaging techniques and computational tools to extract as much information as possible, without the limitations of human perception or bias.

The advances of Carlton et al. (7) have impact across all domains of cell biology. They show it is possible to improve the general design of the light microscope, and in so doing, provide much-improved image data. Future modifications that adapt OMX to single molecule imaging (e.g., PALM and STORM) should be possible soon. Moreover, they suggest that the illumination levels used in many live cell imaging experiments may in fact be doing significant damage to cells, and this should be considered when interpreting results from these assays. Finally, they demonstrate that, although imaging conditions consistent with cell viability may produce signal-limited data, computational techniques like denoising can recover objects that are otherwise invisible. This final insight may be their most important, because it applies to all live cell imaging experiments, recorded on any imaging platform. Denoising then joins a growing list of computational tools that reveal signals invisible to the human eye in modern biology.

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